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Molecular and serological detection of *Ehrlichia* spp. in cats on São Luís Island, Maranhão, Brazil

Detecção molecular e sorológica de *Ehrlichia* spp. em gatos da ilha de São Luís, Maranhão, Brasil

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Abstract

Ehrlichiosis is a tick-borne disease that affects both humans and animals. The few existing reports on ehrlichiosis in Brazilian cats have been based on observation of morulae in leukocytes and, more recently, on molecular detection of *Ehrlichia* sp. In this study, we assessed occurrences of *Ehrlichia* sp. in the blood of 200 domestic cats in São Luís, Maranhão. Of the 200 animals tested, 11 (5.5%) were seropositive for *Ehrlichia* sp. and two (1%) were positive for *Ehrlichia* sp. in PCR. We also performed DNA sequence alignment to establish the identity of the parasite species infecting these animals, using the gene 16S rRNA. One cat presented infection with *Ehrlichia* sp. with 98% identity with *E. canis*, and another cat infected with *Ehrlichia* sp. showed 97% identity with *E. chaffeensis*. This is the first study on molecular detection of *Ehrlichia* sp. among domestic cats in São Luís, Maranhão.

Keywords: Cats, *Ehrlichia* sp., serology, PCR, Brazil.

Resumo

Erliquiose é uma enfermidade transmitida por carrapatos que afeta seres humanos e animais. Os poucos relatos de erliquiose em gatos, no Brasil, são baseados na observação de mórulas em leucócitos e, mais recentemente, na detecção molecular de *Ehrlichia* sp. Neste estudo, foi avaliada a ocorrência de *Ehrlichia* sp. no sangue de 200 gatos de São Luís, Maranhão. Dos 200 animais testados, 11 (5,5%) foram soropositivos para *Ehrlichia* sp. e dois (1%) foram positivos na PCR para *Ehrlichia* spp. O alinhamento de sequências de DNA baseado no gene 16S rRNA foi conduzido para estabelecer a identidade da espécie de parasito que infectou estes animais. Um gato apresentou infecção por uma espécie de *Ehrlichia* sp. com 98% de identidade com *E. canis*; e outro mostrou-se infectado por *Ehrlichia* sp. com 97% de identidade com *E. chaffeensis*. Este estudo traz a primeira detecção molecular de *Ehrlichia* sp. em gatos de São Luís, Maranhão.

Palavras-chave: Gatos, *Ehrlichia* sp., sorologia, PCR, Brasil.

Introduction

Ehrlichiosis is an emerging tick-borne disease that affects both humans and animals (WALKER; DUMLER, 1996). The clinical signs and abnormal laboratory findings relating to ehrlichiosis are similar in felids and canids (ALMOSNY et al., 1998). Suggestive morulae from agents in the family Anaplasmataceae have been detected among cats in France (BEAUFILS et al., 1999), Sweden (BJÖERSDORFF et al., 1999) and Italy (TARELLO, 2005). Some serological studies have demonstrated that antibodies against agents in the family Anaplasmataceae are present in cat serum (MATTHEWMAN et al., 1996; AGUIRRE et al., 2004; ORTUÑO et al., 2005; SOLANO-GALLEGO et al., 2006). The presence of DNA from *Ehrlichia* sp. has been detected among domestic cats in the United States (BREITSCHWERDT et al., 2002), Taiwan (YIN-CHIACHUN et al., 2003), Spain (TABAR et al., 2007) and Brazil (OLIVEIRA et al., 2009). DNA from *Anaplasma phagocytophilum* has been detected in the blood of domestic cats in Sweden (BJÖERSDORFF et al., 1999) and the United States (LAPPIN et al., 2004).

Otherwise, very little is known about the presence of ehrlichiosis-based pathogens in Brazilian cats. In Brazil, the first occurrence of feline ehrlichiosis was reported in 1998, in which morulae of mono and polymorphonuclear leukocytes were observed in

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a cat, with clinical and laboratory findings similar to those of canine ehrlichiosis (ALMOSNY et al., 1998). Recently, DNA from *E. canis* was detected in blood samples from cats in the state of Minas Gerais (OLIVEIRA et al., 2009). A high titer of antibodies against *E. canis* was detected in a free-ranging puma in Brazil (FILONI et al., 2006). Moreover, DNA from *Ehrlichia* spp. and antibodies against *E. canis* have been detected in blood samples from wild felids in the state of São Paulo and in Brasília (ANDRÉ et al., 2010). The present work aimed to detect the presence of *Ehrlichia* spp. in blood samples from cats in São Luís, Maranhão, Brazil, using molecular and serological techniques.

Material and Methods

Between October 2008 and January 2009, EDTA whole blood and serum samples were collected from 200 domestic cats in São Luís, Maranhão, Brazil. The blood and serum samples were stored at –20 °C.

The presence of anti-*Ehrlichia canis* antibodies in the serum samples from each animal was detected by means of the indirect immunofluorescent assay (IFA). Antigen slides were removed from storage and allowed to thaw at room temperature for 30 minutes. Ten microliters of twofold dilutions of serum at 1:64 (cut-off) were placed in wells on antigen slides. Antigens of *Ehrlichia canis* were obtained by culturing DH82 cells infected with *E. canis* (Jaboticabal strain) at the Immunoparasitology Laboratory, UNESP (AGUIAR et al., 2007b). Known positive canine serum (titer 1:2,560) was obtained from a symptomatic dog with ehrlichiosis at Governador Laudo Natel Veterinary Hospital, UNESP, Jaboticabal, São Paulo. When highly positive serum was obtained from a feline (titer of 2,560), it was used as a positive control. Test serum samples that had been analyzed using a canine positive control were reprocessed again using a feline positive control. The negative control serum sample was obtained from a cat that had not been exposed to this agent, according to negative PCR and IFA results.

The slides were incubated at 37 °C in a moist chamber for 30 minutes, washed three times in PBS (pH 7.2) for 5 minutes, and air dried at room temperature. Anti-cat conjugate (dilution of 1:100) for feline samples and anti-dog conjugate for controls (dilution of 1:80) were diluted in accordance with the manufacturer's instructions and then added to each well. These slides were incubated again, washed, dried and overlain with buffered glycerin (pH 8.7), covered with glass coverslips and examined using a fluorescence microscope (NAKAGHI et al., 2008).

DNA was extracted from 200 µL of EDTA whole blood from felines, using the QIAamp DNA blood mini-kit (QIAGEN, Valencia, California, USA), in accordance with the manufacturer's instructions. Each sample of extracted DNA was used as a template in a nested PCR with genus-specific primers (478 bp) and species-specific primers for *Ehrlichia canis* (358 bp) (MURPHY et al., 1998) and *E. chaffeensis* (410 bp) (KOCAN et al., 2000). A positive DNA control for *Ehrlichia canis* was obtained from a dog that had been experimentally infected with the Jaboticabal *E. canis* strain. The positive control for *Ehrlichia chaffeensis* was kindly supplied by J. Stephen Dumler, Department of Pathology, Johns Hopkins School of Medicine, Baltimore, Maryland, USA. A negative domestic cat blood sample was used as the negative control. The PCR and nested PCR amplifications were performed in a Gradient Cycler (Perkin-Elmer™ model PT-200).

PCR amplicons were ligated into pGEM-T Easy Vector followed by transformation of DH10B *Escherichia coli* using the pGEM cloning kit (Promega, Madison, Wisconsin, USA), in accordance with the manufacturer's instructions. The resulting clones underwent blue/white colony screening. Plasmid DNA from the positive clones was isolated by means of the alkaline lysis method (SAMBROOK; RUSSEL, 2001) and was subjected to sequence determination (ABI Prism 310 Genetic Analyzer, Applied Biosystems/Perkin Elmer, Foster City, California, USA). Consensus sequences were obtained using the CAP3 software (http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py) for subsequent phylogenetic analysis and the BLAST tool was used to ascertain similarities with GenBank sequences. The CLUSTAL W (THOMPSON et al., 1994) and MEGA (KUMAR et al., 2004) software was used for alignment and phylogenetic analysis, respectively. The distance-based neighbor-joining method was used to build the phylogenetic tree (SAITOU; NEI, 1987) using the Kimura-2-parameter model. The bootstrap test with 1000 replications was used to estimate the confidence of branching patterns on the neighbor-joining tree (FELSENSTEIN, 1985).

Results and Discussion

Eleven (5.5%) of the 200 cats tested were seropositive for *E. canis* antigens, according to IFA. The antibody titers ranged from 64 (cut-off) to 512 for *E. canis*. Three animals showed antibody titers of 64 and seven animals were seroreactive at a dilution of 1:160. Only one animal showed antibody titers of 512.

Two (1%) of the 200 samples were positive for *Ehrlichia* sp. PCR based on 16S rRNA. One sample (cat#45) was positive for *E. canis* nPCR and another one (cat#211) for *E. chaffeensis* nPCR. Both of the PCR-positive animals were negative in the serological test. DNA sequencing using the 16S rRNA gene showed that the *Ehrlichia* sp. DNA obtained from cat#45 (GenBank access number JN123433) was closely related (98.0% identity) to *E. canis* from dogs in Tunisia (EU781695), Taiwan (EU143637) and Italy (EU439944). The *Ehrlichia* sp. DNA obtained from cat#211 (GenBank access number JN123434) was closely related (97.0% identity) to *E. chaffeensis* isolated in Arkansas (AF416764), *Ehrlichia* sp. from *Boophilus microplus* in Tibet (AF414399) and *Ehrlichia* sp. from deer in Japan (AB454074). While the *Ehrlichia* sp. isolate from cat#45 clustered together with *E. canis* isolates, the *Ehrlichia* sp. from cat#211 clustered together with *E. chaffeensis* isolates (Figure 1).

To our knowledge, the present study is the first to show the presence of DNA from *Ehrlichia* spp. among cats in the state of Maranhão, Brazil. *Ehrlichia* DNA has been detected among cats in the United States (BREITSCHWERDT et al., 2002), Spain (TABAR et al., 2007) and Brazil (OLIVEIRA et al., 2009). *Ehrlichia* spp. DNA and antibodies to this agent have been detected in blood samples from wild felids that were kept in captivity in the state of São Paulo and the Brazilian Federal District (ANDRÉ et al.,

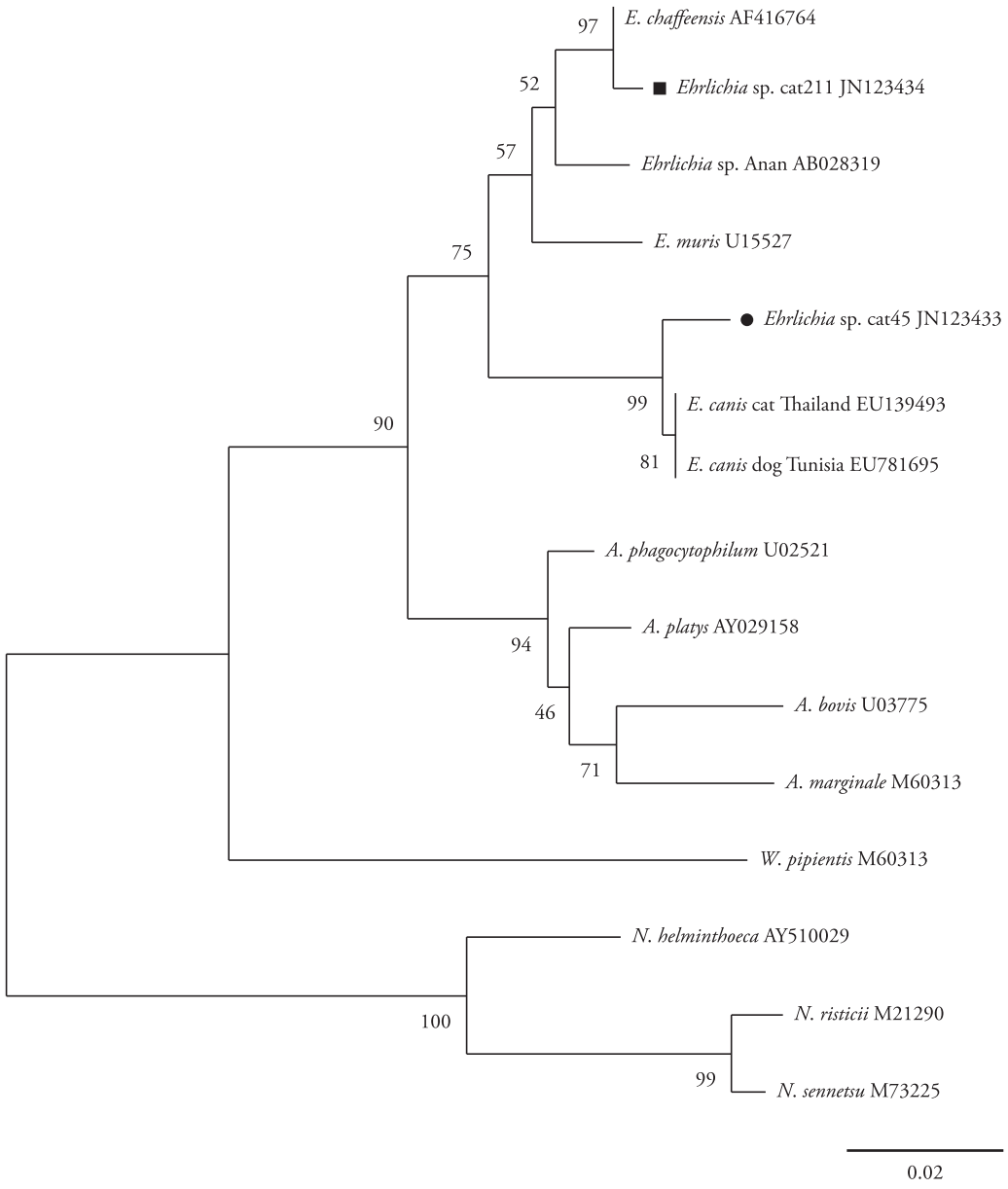

Figure 1. Phylogenetic position of *Ehrlichia* sp. isolates from Brazilian domestic cats on São Luís island, based on 16S rRNA sequences (300 bp). The tree was constructed using the neighbor-joining method and the numbers on the tree indicate bootstrap values for the branch points. Accession numbers and place of origin of the isolates are shown beside the sequence names.

2010). Furthermore, DNA from *Anaplasma phagocytophilum* has been detected among cats in Sweden (BJÖERSDORFF et al., 1999) and the United States (LAPPIN et al., 2004).

The low percentage of positive animals in the present study and other studies can possibly be explained by considering the fact that cats are more resistant to *Ehrlichia* infection than dogs are, and they interact differently with the tick vector. Most cats rapidly remove ticks when they become infested, and thus the minimum tick attachment time of 24-48 hours that is thought likely to be required for transmission of most tick-transmitted infections may not be achieved (KIDD; BREITSCHWERDT, 2003). It is also possible that cats have lower copy numbers of *Ehrlichia* DNA than shown by dogs, thereby resulting in false negative results (EBERHARDT et al., 2006). Moreover, these results show that *Ehrlichia* infections in cats are uncommon.

The percentage of seropositive animals in the present study was lower than that found in other studies on domestic cats (MATTHEWMAN et al., 1996; AGUIRRE et al., 2004; ORTUÑO et al., 2005; SOLANO-GALLEGO et al., 2006). The observed low antibody titers may result from a low humoral immune response to *Ehrlichia* sp. among cats, or may result from cross-reactivity with other Anaplasmataceae species (ORTUÑO et al., 2005). The occurrence of positive PCR and negative serological results suggests that genes from other organisms that are closely related to *E. canis* may also have been amplified, but were distinct enough not to induce cross-reacting antibodies (EBERHARDT et al., 2006).

Although it has been suggested that feline ehrlichiosis is transmitted by ticks, the transmission mechanism still remains imperfectly understood (SHAWN, 2001). In Brazil, *E. canis* is transmitted to dogs by the tick *Rhipicephalus sanguineus* (AGUIAR et al., 2007a). Although *E. chaffeensis* DNA has been detected in Brazilian marsh deer, there is no information about its transmission route (MACHADO et al., 2006). Although the samples for the present study were collected during the summer, ticks were found attached in only one of the sampled animals (*Rhipicephalus sanguineus* ticks in one cat that was negative to both PCR and serological tests; data not shown). It should be noted that exposure to vectors among felids is less frequent than among dogs. Alternatively, felids may remove the vectors before hemoparasite transmission occurs (LAPPIN et al., 2006). At present, the exposure route to *E. canis* among the cats studied here is unknown.

The role of cats in the epidemiology of ehrlichiosis is unknown. It has been suggested that felids are more resistant to infection than are dogs (LAPPIN et al., 2006). The presence of *Ehrlichia* sp. closely related to *E. chaffeensis* in one cat in the present study, along with reports of antibodies to *E. chaffeensis* both in humans (CALIC et al., 2004; DA COSTA et al., 2005, 2006) and in dogs (GALVÃO et al., 2002) in the state of Minas Gerais, Brazil, and the molecular detection of this agent in Brazilian marsh deer (*Blastocerus dichotomus*) (MACHADO et al., 2006), shows that there is a need for more studies on the zoonotic potential represented by domestic and wild animals in Brazil, taking into consideration the existence of human monocytic ehrlichiosis.

The present work shows that Brazilian cats have been exposed to *Ehrlichia* sp. infection. To determine whether this finding represents a real threat to the health of these animals, further studies are needed. To our knowledge, this is the first study on molecular detection of *Ehrlichia* sp. among cats in the state of Maranhão.

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